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# Herpesviral ICP0 Protein Promotes Two Waves of Heterochromatin Removal on an Early Viral Promoter during Lytic Infection

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**ABSTRACT** Herpesviruses must contend with host cell epigenetic silencing responses acting on their genomes upon entry into the host cell nucleus. In this study, we confirmed that unchromatinized herpes simplex virus 1 (HSV-1) genomes enter primary human foreskin fibroblasts and are rapidly subjected to assembly of nucleosomes and association with repressive heterochromatin modifications such as histone 3 (H3) lysine 9-trimethylation (H3K9me3) and lysine 27-trimethylation (H3K27me3) during the first 1 to 2 h postinfection. Kinetic analysis of the modulation of nucleosomes and heterochromatin modifications over the course of lytic infection demonstrates a progressive removal that coincided with initiation of viral gene expression. We obtained evidence for three phases of heterochromatin removal from an early gene promoter: an initial removal of histones and heterochromatin not dependent on ICP0, a second ICP0-dependent round of removal of H3K9me3 that is independent of viral DNA synthesis, and a third phase of H3K27me3 removal that is dependent on ICP0 and viral DNA synthesis. The presence of ICP0 in transfected cells is also sufficient to promote removal of histones and H3K9me3 modifications of cotransfected genes. Overall, these results show that ICP0 promotes histone removal, a reduction of H3K9me3 modifications, and a later indirect reduction of H3K27me3 modifications following viral early gene expression and DNA synthesis. Therefore, HSV ICP0 promotes the reversal of host epigenetic silencing mechanisms by several mechanisms.

**IMPORTANCE** The human pathogen herpes simplex virus (HSV) has evolved multiple strategies to counteract host-mediated epigenetic silencing during productive infection. However, the mechanisms by which viral and cellular effectors contribute to these processes are not well defined. The results from this study demonstrate that HSV counteracts host epigenetic repression in a dynamic stepwise process to remove histone 3 (H3) and subsequently target specific heterochromatin modifications in two distinct waves. This provides the first evidence of a stepwise reversal of host epigenetic silencing by viral proteins. This work also suggests that targets capable of disrupting the kinetics of epigenetic regulation could serve as potential antiviral therapeutic agents.

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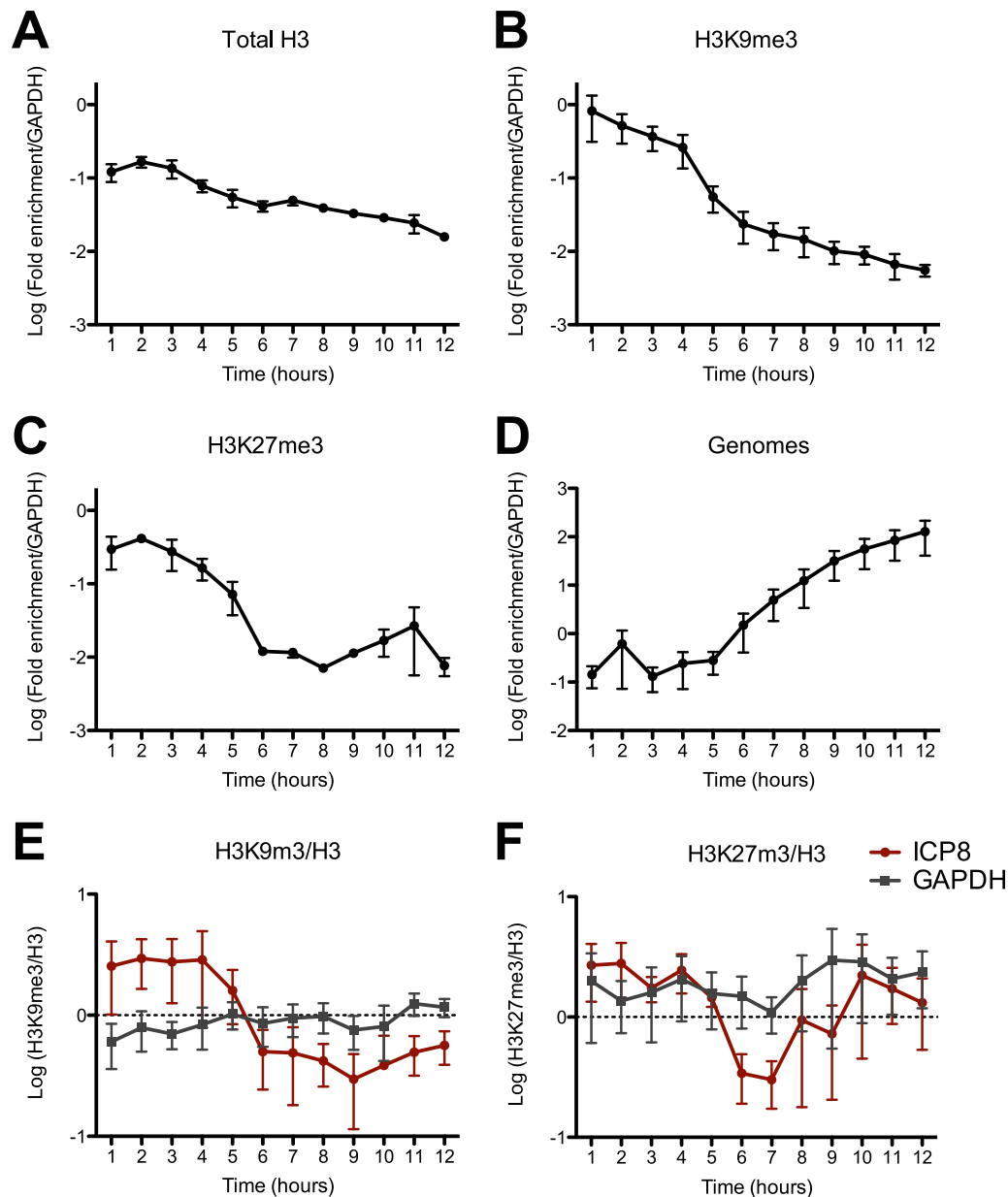
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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

The assembly of histones onto incoming naked DNA by the recipient cell appears to have evolved in eukaryotic cells as an intrinsic response to silence foreign DNA through compaction into repressive heterochromatin (1, 2), which serves to silence the foreign DNA and prevent the expressed gene products from affecting the host cell. Chromatin has a dynamic structure that serves an important role in regulating gene expression. Chromatin structure can be modified by histone chaperones facilitating the selective addition and removal of nucleosome components, chromatin remodelers modulating nucleosome density and positioning, or a variety of chromatin-modifying enzymes directing the addition or removal of specific covalent modifications to or from histone tails. These factors are necessary to establish and maintain distinct forms of chromatin that can be interpreted by chromatin “readers” to impact transcription of genes. Chromatin

that is densely compacted with regularly spaced nucleosomes through association with heterochromatin protein 1 (HP1) and covalent modifications at histone tails such as histone 3 (H3) lysine 9-trimethylation (H3K9me3) and lysine 27-trimethylation (H3K27me3) is not accessible to RNA polymerase II and transcription factors and thus is epigenetically silenced (3, 4). Conversely, chromatin that is less densely packed with nucleosomes and/or enriched in modifications such as H3 lysine 9-acetylation (H3K9ac) or lysine 4-trimethylation (H3K4me3) is accessible to RNA polymerase II and transcription factors for transcription (5). Histone-mediated interactions also regulate many processes, including transcriptional response to signaling (6–8), mRNA splicing (9), DNA repair (10), and positioning of chromatin within the three-dimensional nuclear space (11).

During productive infection, herpes simplex virus (HSV)



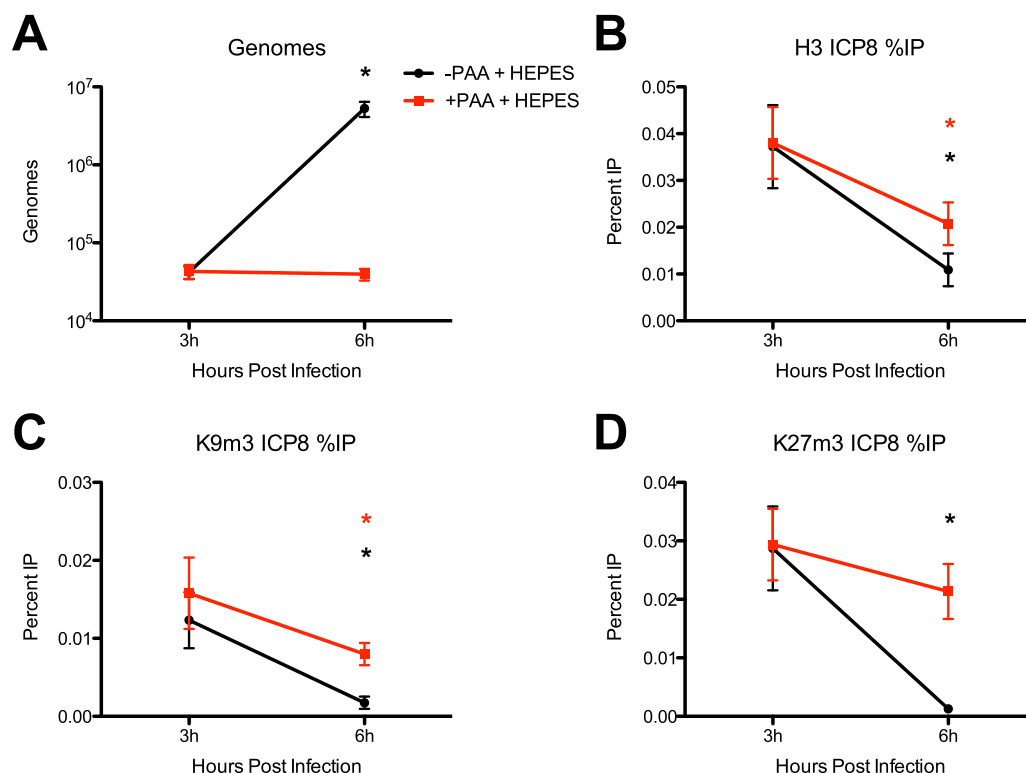
**FIG 1** Kinetic analyses of chromatin association with HSV-1 during lytic infection. (A to C) Chromatin immunoprecipitation assay of histones and heterochromatin on the *ICP8* promoter of WT HSV-1 KOS was performed for 12 h following infection of human foreskin fibroblast cells at 3 PFU/cell. ChIP was carried out using antibodies specific for total H3 (A), H3K9me3 (B), and H3K27me3 (C). The amounts of input and percentage of immunoprecipitated DNA were measured by quantitative PCR using primers specific for the *ICP8* promoter and cellular GAPDH pseudogene. ChIP data are presented as the fold enrichment of immunoprecipitated *ICP8* DNA relative to GAPDH gene DNA. (D) Chromatin inputs were used to calculate relative genome copy numbers. (E and F) The relative proportions of H3 bearing heterochromatin modifications were calculated from the percentage of DNA immunoprecipitated with antibodies specific for (E) H3K9me3 or (F) H3K27me3 modifications normalized to the percentage of DNA immunoprecipitated with an antibody specific to total H3. The mean values and standard errors of the means of results of at least three independent experiments are shown.

genes are expressed in an ordered cascade of immediate-early (IE), early (E), and late (L) genes (12, 13), in a process dependent on viral and cellular transcriptional machinery (14, 15). This was originally thought to be the result of a series of gene activation steps (13), but more recent studies have shown that reversal of epigenetic silencing is also involved in the sequential activation of viral gene expression (16–18).

The double-stranded DNA genome of HSV-1 enters the host cell free of histones (19–22). Histones are known to be

loaded rapidly on HSV-1 E and L promoters and then removed (17, 19). Histone H3K9me3 is also present at high levels at early times that decrease on the IE *ICP0* gene promoter (23), consistent with removal of heterochromatin on IE genes. Acetylation of H3 on viral IE and E gene promoters increases during infection, consistent with the addition of euchromatin markers (24). The changes in IE gene chromatin require VP16 and associated host proteins (25, 26).

Each round of lytic infection must contend with the cellular



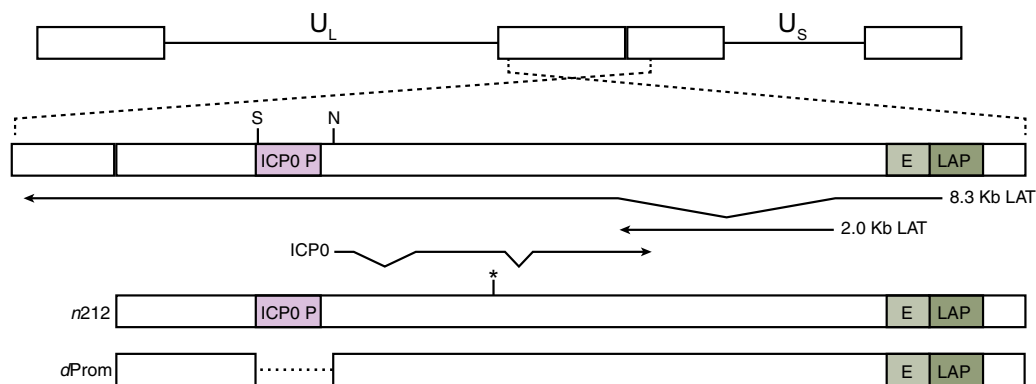
**FIG 2** Effect of viral DNA synthesis on removal of histones and chromatin modifications. HFF cells were treated with the viral polymerase inhibitor PAA or mock treated and infected with WT HSV-1 at 3 PFU/cell to evaluate if histone removal was due to vDNA synthesis. Cell lysates were prepared at 3 and 6 hpi. (A) Relative viral genome copy numbers were determined by qPCR of viral *ICP8* DNA normalized to cellular GAPDH gene DNA. (B to D) ChIP assays were carried out with antibodies specific for total H3 (B), H3K9me3 (C), and H3K27me3 (D). The fraction of *ICP8* DNA immunoprecipitated was measured by qPCR, and data are shown as the means and standard errors of the means for results from at least three independent experiments. Samples with mean values that varied significantly from 3 to 6 hpi ( $P < 0.05$ , paired Student's  $t$  test) are indicated (\*).

chromatinization response to prevent silencing, initiate the ordered cascade of lytic gene expression, and facilitate production of infectious progeny. Previous studies have demonstrated that, as lytic infection of HSV-1 progresses, viral promoters associate with unstable nucleosomes that are enriched for histones bearing activating modifications (17, 27–29). Epigenetic changes can occur as both cause and consequence of transcription, and their specific roles and relative levels of importance to viral infection have generated some debate (30, 31). It has been demonstrated that efficient productive HSV-1 infection requires cellular chromatin modifiers such as the SNF2H chromatin remodeler for IE viral gene expression (32), while the CHD3 chromatin remodeler has been implicated in initial repression of gene expression (33). Additional factors such as the histone chaperone human antisilencing factor 1a (Asf1a) and the exchange of histone variants have also been implicated in epigenetic regulation of HSV-1 (34, 35).

Viral proteins, including VP16 and ICP0, have also been implicated in chromatin regulation critical for lytic infection. The VP16 tegument protein activates IE gene expression through interaction with cellular factors HCF-1 and Oct-1 and the recruitment of chromatin methylation modifiers, including SETD1A methyltransferase, LSD1 (an H3K9 di- or monodemethylase), and JMJD2 (an H3K9me3 demethylase) (23). In addition, VP16 can recruit nucleosome remodelers such as BRG1 and BRM, as well as histone acetyltransferases (HATs) such as p300 and CBP, to viral IE promoters (25, 31). Accordingly, in the absence of VP16, IE

gene promoters are associated with increased histone accumulation, and E gene promoters are associated with reduced histone acetylation (25).

The HSV IE ICP0 protein, a multifunctional E3 ubiquitin ligase and potent gene transactivator in infected (36, 37) and cotransfected (38–41) cells, is required for expression of early proteins such as ICP8 (36, 37, 42). During lytic infection of HeLa cells, ICP0 was found to promote the acetylation of viral chromatin and to reduce total histone accumulation on the viral genome (17). This argues that one mechanism by which ICP0 promotes viral gene expression is through regulation of chromatin on the viral genome by limiting histone association and/or promoting association with active euchromatin. ICP0 promotes the acetylation of viral chromatin by preventing the removal of histone acetylations by binding to CoREST and disrupting histone deacetylase 1 (HDAC1) binding to the HDAC1/CoREST/LSD1/REST (HCLR) repressor complex, ultimately causing relocalization of HCLR components to the cytoplasm later in infection (16, 43–45). During infection, stabilization and recruitment of CLOCK H3/H4 acetyltransferases facilitate expression of viral genes, and CLOCK overexpression can compensate for defects in ICP0-deficient viruses (46, 47). ICP0 also promotes the degradation of nuclear domain 10 (ND10) components, namely, PML, Sp100, and Daxx, which are thought to be involved in epigenetic silencing of the viral genome (48). However, the specific mechanism of ND10 silencing of the HSV genome remains to be defined. An additional



**FIG 3** HSV-1 genomic map of the *ICP0* region. The unique short regions ( $U_S$ ) and unique long regions ( $U_L$ ) of HSV-1 flanked by inverted repeats are shown here. A view of the *ICP0* region, which encodes *ICP0*, the antisense primary 8.3-kb *LAT* transcript, and the stable 2-kb *LAT* intron, is magnified. The *dProm* mutant virus has a 711-bp deletion between the *NcoI* (N) and *StuI* (S) sites depicted on this map. The *n212* mutant contains a nonsense mutation in codon 212 in exon 2 of the *ICP0* transcript (indicated by a star).

restriction factor, IFI16, can promote accumulation of silencing H3K9me3 modifications on the viral genome and other foreign unchromatinized DNA (49); however, *ICP0* promotes the degradation of IFI16 during infection with HSV-1 (50). These studies highlight the importance and diversity of targets of *ICP0* in lytic infection associated with its ability to prevent chromatin-mediated silencing.

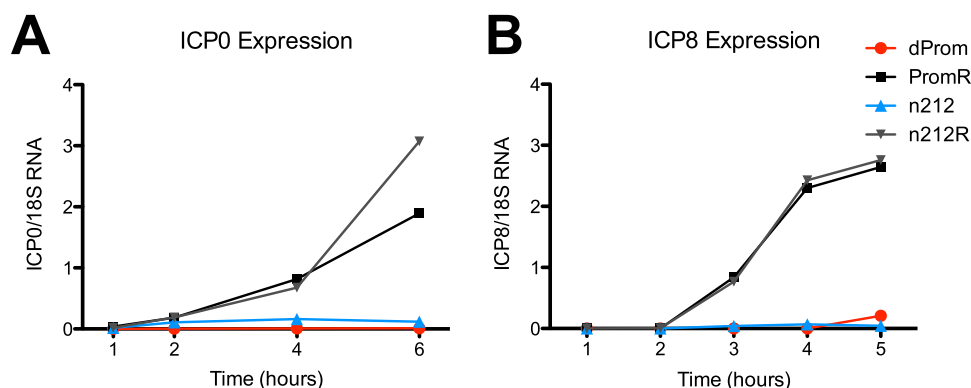
Previous studies of HSV-1 chromatin during lytic infection have focused mainly on IE gene promoters. Thus, studies have identified H3K9me3 heterochromatin associated with viral genomes during initial acute infection (26, 51) but excluded from replication compartments (52). Additionally, a reduction of H3K9me3 heterochromatin at the *ICP0* promoter coincided with the expression of IE genes (26). However, little was known about the effects of heterochromatin on E viral promoters. In this study, we examined the kinetics of histone association and heterochromatin modifications H3K9me3 and H3K27me3 on an E gene promoter during lytic infection of primary human foreskin fibroblasts (HFFs). Additionally, we evaluated the role of the *ICP0* protein by studies of two *ICP0*-deficient viruses that either fail to transcribe the *ICP0* gene or express only a truncated *ICP0* protein. We found that, during lytic infection, viral genomes were initially associated with repressive hetero-

chromatin modifications followed by a stepwise restructuring of chromatin at viral gene promoters. The *ICP0*-null viruses were unable to effectively reverse host-mediated epigenetic silencing, indicating that *ICP0*, either directly or indirectly, impacts the level of associated histones and heterochromatin markers to facilitate viral gene expression.

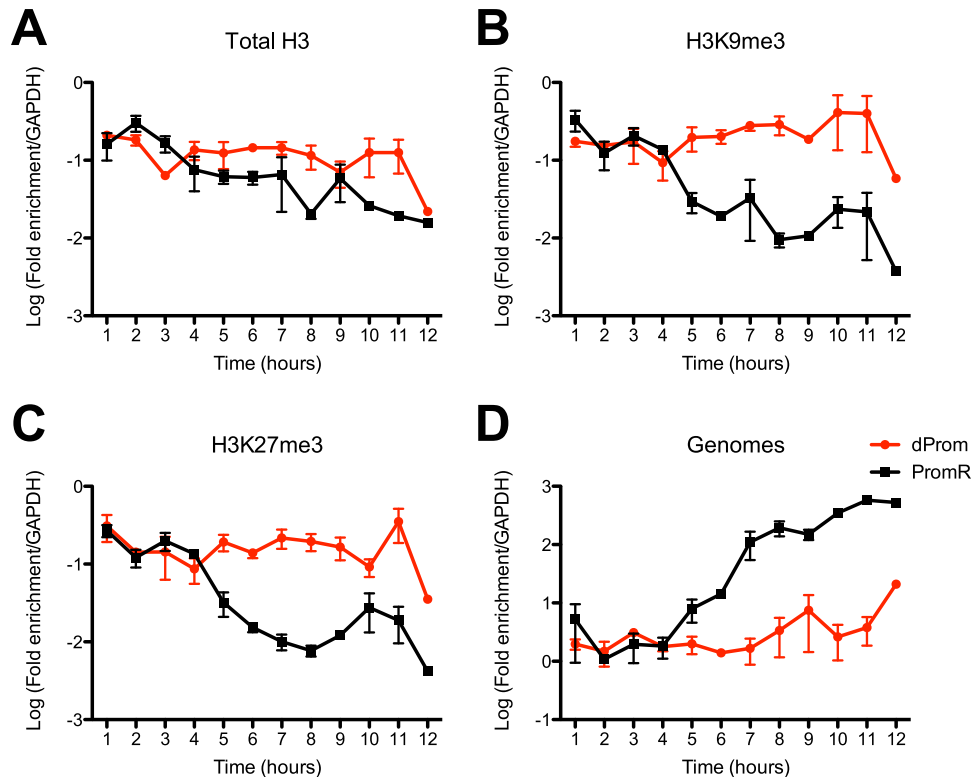
## RESULTS

**Kinetic analysis of HSV-1 chromatin reveals early association with histones and heterochromatin modifications during lytic infection.** To examine the kinetics of chromatin association with an HSV E gene promoter, we infected HFF cells at 3 PFU/cell with wild-type (WT) KOS strain HSV-1 and collected cells at every hour postinfection (hpi) for 12 h. Cells were subsequently processed, and chromatin was analyzed by immunoprecipitation with antibodies specific for H3, H3K9me3, or H3K27me3. We performed chromatin immunoprecipitation (ChIP) analysis using quantitative PCR (qPCR) with specific primers to measure the extent of association with histones and specific histone modifications at the viral *ICP8* E gene promoter normalized to the proportion of cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene DNA immunoprecipitated from the same reaction.

Over 12 h of infection, the levels of total histone and hetero-



**FIG 4** Viral transcript accumulation during lytic infection. HFF cells were infected at 3 PFU/cell with the PromR or *n212R* *ICP0*<sup>+</sup> viruses or with the *dProm* or *n212* *ICP0*-null mutant viruses. Total RNA was isolated from cell lysates and quantified using quantitative reverse transcription-PCR (qRT-PCR). Viral transcripts are expressed normalized to cellular 18S rRNA.



**FIG 5** Kinetic analyses of histones and heterochromatin on the *ICP8* promoter in an *ICP0* mutant *dProm* virus relative to its rescued virus *PromR* during lytic infection. (A to C) HFF cells were infected with *dProm* or *PromR* viruses at 3 PFU/cell. Cells were collected hourly for 12 h, and chromatin was processed for immunoprecipitation with antibodies specific for total H3 (A), H3K9me3 (B), or H3K27me3 (C). ChIP results are presented as the proportion of the *ICP8* promoter DNA immunoprecipitated relative to cellular GAPDH gene DNA measured by qPCR. (D) Relative viral genome copy numbers were calculated from input chromatin measurement of viral *ICP8* DNA normalized to cellular GAPDH gene DNA. The means and standard errors of the means of results of at least three independent experiments are shown.

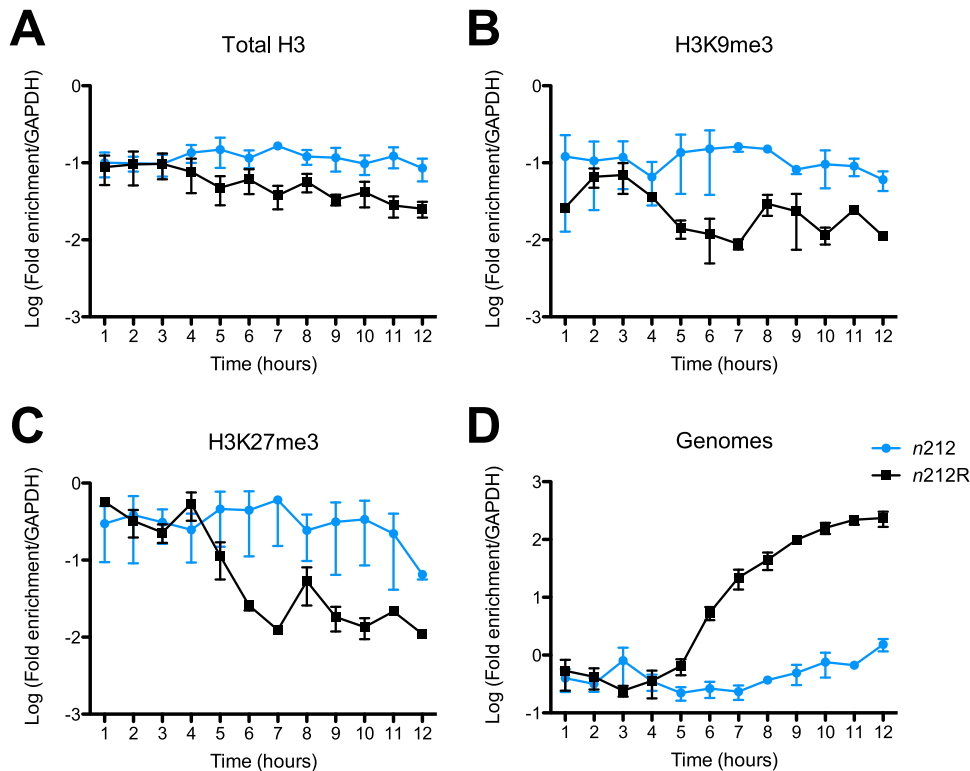
chromatin modifications remained constant at the cellular GAPDH gene promoter (not shown) but decreased dramatically at the viral *ICP8* promoter (Fig. 1). We observed that the *ICP8* gene promoter accumulated the highest levels of H3 and of heterochromatin modifications H3K9me3 and H3K27me3 at 1 to 2 hpi (Fig. 1A to C). Overall, relative to the initial peak of accumulation, the *ICP8* promoter showed a 10-fold reduction in the levels of total H3 and a 50- to 100-fold reduction in the H3K9me3 and H3K27me3 heterochromatin modifications, respectively, during the following 10 h. To identify the time periods when chromatin levels changed most dramatically, we calculated the fold change for each hour relative to the previous hour (see Fig. S1 in the supplemental material). We observed a significant reduction in total H3 before 4 hpi, in H3K9me3 at 3 to 7 hpi, and H3K27me3 at 5 hpi (see Fig. S1). Interestingly, while the absolute levels of total H3, H3K9me3, and H3K27me3 changed rapidly during the first 4 hpi, the relative proportion of H3 bearing K9me3 or K27me3 remained constant (Fig. 1E and F). This resulted in a reduced proportion of histones bearing H3K9me3 or H3K27me3 modifications at the *ICP8* promoter relative to cellular GAPDH gene sequences by 6 hpi.

**Removal of nucleosomes and H3K9me3 heterochromatin modifications occurred independently of viral DNA synthesis.** The time period of the most prominent heterochromatin removal on the *ICP8* promoter relative to total H3 removal occurred after 4 hpi, coinciding with the initiation of viral DNA synthesis

(Fig. 1D). Therefore, we wanted to determine whether viral DNA synthesis or potential dilution of histones and heterochromatin modification by newly synthesized genomes or both were responsible for their apparent reduction. We infected HFF cells at 3 PFU/cell with WT HSV-1 in the presence or absence of a viral DNA polymerase inhibitor, sodium phosphonoacetate (PAA). Control-treated infections demonstrated a significant increase in viral genomes between 3 and 6 hpi, while PAA-treated cells showed no increases in viral genome copy number (Fig. 2A). We performed ChIP analysis on samples isolated at 3 and 6 hpi with antibodies specific for H3, H3K9me3, and H3K27me3 as described above. We detected significant decreases in the levels of total H3, H3K9me3, and H3K27me3 in control cells between 3 and 6 hpi (Fig. 2B to D). The levels of H3 and H3K9me3 (Fig. 2B and C) decreased significantly in PAA-treated cells; however, H3K27me3 levels did not change significantly between 3 and 6 hpi (Fig. 2D) in the PAA-treated cells. These results argued that removal of histones and H3K9me3 occurred independently of viral DNA synthesis. Surprisingly, however, the H3K27me3 modification was not removed in cells treated with PAA, indicating that removal of H3K27me3 was dependent on viral DNA synthesis or the participation of a viral L gene product.

**Construction and analysis of HSV-1 ICP0 mutant viruses.** We wanted to determine whether ICP0 was facilitating the dynamic reversal in chromatin association during lytic infection to reduce chromatin silencing and promote viral gene expression.



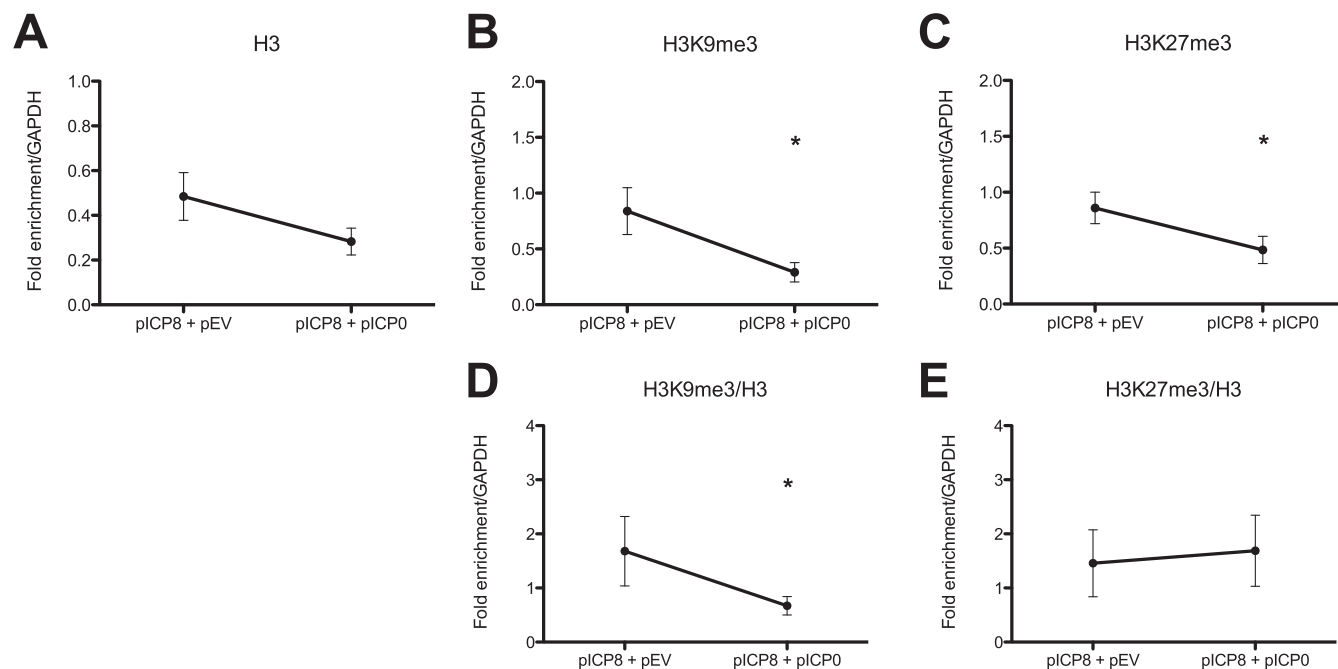


**FIG 6** Histone and heterochromatin association on an *ICP0* mutant *n212* virus compared to the rescued *n212R* virus during lytic infection. HFF cells were infected with *n212* or *n212R* viruses at 3 PFU/cell. (A to C) Cells were collected hourly for 12 h, and chromatin was processed for immunoprecipitation with antibodies specific for total H3 (A), H3K9me3 (B), and H3K27me3 (C). ChIP results are presented as the proportion of the *ICP8* promoter DNA immunoprecipitated relative to cellular GAPDH gene DNA measured by qPCR. (D) Relative viral genome copy numbers were calculated from input chromatin measurement of *ICP8* DNA normalized to GAPDH gene DNA. The means and standard errors of the means of results of at least three independent experiments are shown.

We tested two independently constructed *ICP0* mutant HSV-1 strains, *n212* and *dProm* (Fig. 3). The *n212* virus (53) contains a nonsense mutation in codon 212 that results in expression of a truncated *ICP0*. We constructed the *n212R* rescued virus as the *ICP0*-positive (*ICP0*<sup>+</sup>) control virus. The *dProm* virus contained a 711-bp deletion of the *ICP0* transcription start site and upstream promoter region that eliminated transcription of the *ICP0* gene (Fig. 4A). Construction of the *dProm* virus and of the cognate rescued virus PromR is described in Materials and Methods. Characterization of these viruses confirmed reduced viral gene expression and replication by the *ICP0*<sup>−</sup> viruses compared to the rescued viruses in HFF cells (see Fig. S2 in the supplemental material), characteristic of *ICP0*-null mutant viruses.

***ICP0* mutant HSV-1 strains show no removal of nucleosomes and heterochromatin.** To study the kinetics and structure of viral chromatin in *ICP0* mutant-infected cells, we infected HFF cells with *n212* or *dProm* virus as well as with the corresponding rescued viruses at 3 PFU/cell. Viral replication and genome accumulation were quantified by measurement of levels of viral DNA normalized to the GAPDH gene. The *ICP0* mutant viruses demonstrated reduced and delayed viral DNA synthesis (Fig. 5D and 6D) and reduced *ICP8* RNA expression (Fig. 4B). ChIP analysis was carried out to measure the histone modifications of total H3 and of H3K9me3 and H3K27me3 associated with the HSV-1 genome. Immunoprecipitated DNA levels were measured by qPCR and expressed as the fraction of the *ICP8* gene promoter sequences

that were immunoprecipitated normalized to the fraction of the GAPDH gene promoter sequences that were immunoprecipitated from the same reaction. We observed that the *n212R* and PromR *ICP0*<sup>+</sup> viruses demonstrated kinetics of initial histone and heterochromatin association and subsequent removal similar to those observed previously with the WT KOS strain virus (Fig. 5A to C and Fig. 6A to C). The *n212* and *dProm* *ICP0*<sup>−</sup> mutant HSV-1 strains accumulated histones and heterochromatin modifications to levels similar to those seen with *ICP0*<sup>+</sup> viral strains at 1 to 2 hpi, but these associations were maintained throughout the 12 hpi. While the trends in data were reproducible, the small sample size and the scatter resulted in statistically insignificant changes. Therefore, we combined the values for samples from 1 to 4 hpi, 5 to 8 hpi, and 9 to 12 hpi and tested for significance using a two-tailed Wilcoxon ranked-sum test. Combined analysis of the data from the first 4 hpi did not show statistically significant differences between *ICP0*<sup>+</sup> and *ICP0*<sup>−</sup> viruses. However, from 5 to 8 hpi, the *ICP0* mutant viruses were unable to effectively remove histones and repressive histone modifications, resulting in a significant ( $P < 0.05$ ) difference from infection with *ICP0*<sup>+</sup> viruses. The trend was upheld at late times postinfection but was not statistically significant in *dProm* and PromR infections, which was the result of high variability of the samples taken at 12 hpi, likely resulting from cell death. Overall, these results indicated that *ICP0* promotes removal of histones and heterochromatin during the



**FIG 7** ICP0 is sufficient to reduce chromatin and heterochromatin on transfected *ICP8* gene plasmid. Cells (293T) were cotransfected with pICP8-GFP plasmid and either an empty vector control (pEV) plasmid or the pICP0 plasmid. (A to C) After 48 h, cells were harvested and processed for ChIP with antibodies specific for total H3 (A), H3K9me3 (B), and H3K27me3 (C). ChIP results were measured by qPCR as the proportion of the immunoprecipitated *ICP8* promoter DNA relative to cellular GAPDH gene DNA. The relative proportions of H3 bearing (D) H3K9me3 and (E) H3K27me3 heterochromatin modifications are also presented. Results are shown as the means and standard errors of the means of results of at least three independent experiments. Samples with mean values that varied significantly ( $P < 0.05$ , paired Student's *t* test) are indicated (\*).

progression of lytic infection and facilitates viral early gene transcription and DNA replication.

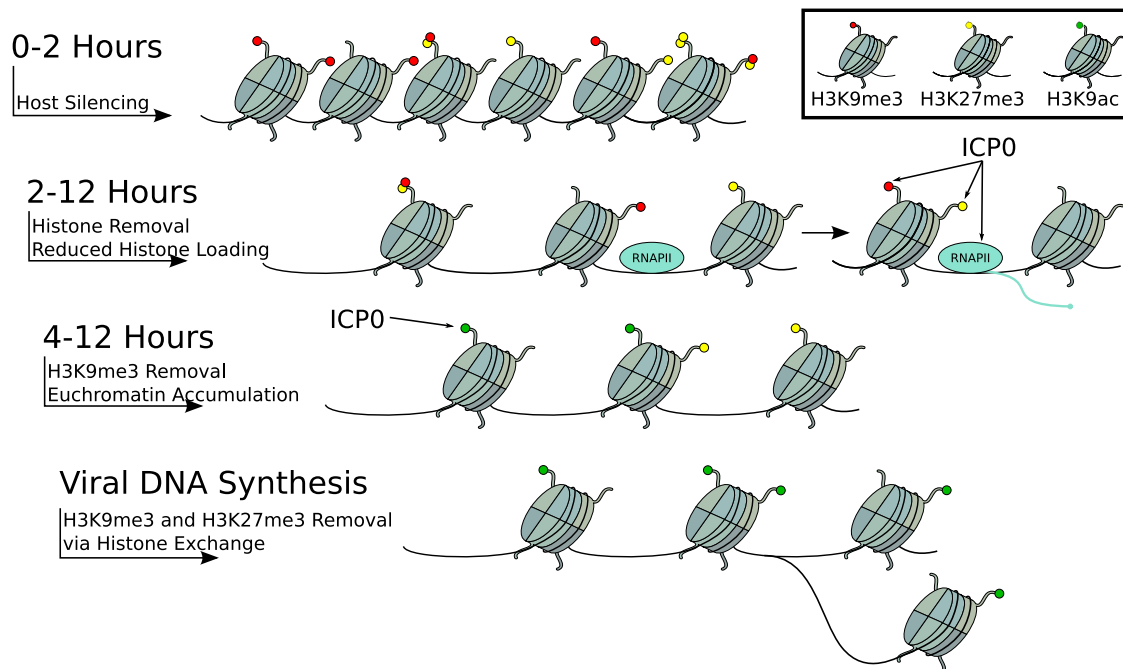
**ICP0 is sufficient for heterochromatin reduction on a cotransfected *ICP8* gene promoter.** ICP0 mutant viruses are defective for viral gene expression during infection of primary HFF cells, so the effect of ICP0 on viral chromatin could be an indirect effect caused by reduced expression of another viral gene product with chromatin modulatory activity or reduced viral DNA synthesis. However, ICP0 expressed alone in transfected cells is capable of transactivating expression of a cotransfected gene (38, 54). Therefore, we wanted to determine whether ICP0 was sufficient to modulate chromatin on the *ICP8* promoter in the absence of other viral gene products, as a potential mechanism of transactivation. We cotransfected 293T cells with a plasmid expressing ICP8-green fluorescent protein (GFP) from the native *ICP8* promoter and either an empty vector plasmid or a plasmid expressing ICP0. As observed previously (38), cotransfection with the ICP0-encoding plasmid promoted expression of ICP8-GFP (results not shown). We performed ChIP assays to determine occupancy of histones and heterochromatin modifications on the *ICP8* promoter and found that ICP0 expression resulted in significantly reduced levels of H3K9me3 and H3K27me3 (Fig. 7B and C). Total H3 occupancy demonstrated a similar trend, but the results were not statistically significant (Fig. 7A). Normalization of H3K9me3 to total H3 showed a significant reduction when ICP0 was expressed (Fig. 7D), but when H3K27me3 was normalized to total H3, ICP0 did not cause a reduction (Fig. 7E). Therefore, ICP0 appeared to cause a specific reduction in H3K9me3 levels but not in H3K27me3 levels. Therefore, we concluded that ICP0 was suffi-

cient to reduce certain heterochromatin modifications at the *ICP8* promoter sequences.

## DISCUSSION

**Epigenetic regulation is a dynamic process that progresses in stages during lytic infection.** Previous studies have shown that histones are loaded and removed rapidly on HSV-1 E and L promoters and that heterochromatin was removed from IE gene promoters (2). However, the kinetics of heterochromatin association with early gene promoters has not been investigated. In this study, we examined the kinetics of total nucleosome and heterochromatin association on a prototype HSV-1 early gene promoter during the lytic infection of primary HFFs (Fig. 8) and found that the accumulation of histones and heterochromatin modifications H3K9me3 and H3K27me3 peaked during the first 2 hpi. Interestingly, the proportion of histones bearing the H3K9me3 modification was initially higher on the viral *ICP8* promoter than on the cellular GAPDH gene. Therefore, the cellular epigenetic silencing response may preferentially assemble histones bearing heterochromatin modifications or rapidly recruit histone methyltransferases. At 2 to 4 hpi, total H3 and heterochromatin levels declined rapidly; however, the ratio of histone to heterochromatin modification remained constant. This argues that the initial antisilencing response by HSV-1 targets nucleosomes indiscriminately, potentially by recruiting histone chaperones or chromatin remodelers. This could expose critical regulatory sequences and initiation sites for the assembly of RNA polymerase II to poise viral genes for transcriptional activation. In the next phase (from 4 to 6 hpi), histone and heterochromatin removal continued, resulting in





**FIG 8** Model of progressive epigenetic regulation of the HSV-1 genome during lytic infection. HSV-1 enters the host cell nucleus and is subjected to host cell repression through the assembly of silenced heterochromatin during the first 2 hpi. After 2 h, HSV-1 reduces epigenetic repression by removing histones to reduce total histone occupancy and heterochromatin levels while maintaining a stable proportion of histones bearing heterochromatin modifications. RNA polymerase II (RNAPII) is recruited and initiates transcription of viral early genes. After 4 h, H3K9me3 and H3K27me3 heterochromatin modifications are specifically targeted for removal, resulting in a lower proportion of histones at viral promoters bearing heterochromatin modifications relative to cellular sequences. H3K27me3 removal is dependent on viral DNA synthesis. ICP0 promotes viral gene transcription, removal of histones and heterochromatin, and accumulation of euchromatic histone modifications.

lower total histone association at the *ICP8* promoter region relative to the *GAPDH* gene sequences. However, while H3 removal slowed, the reduction of H3K9me3 and H3K27me3 heterochromatin modifications increased. Kinetic studies showed parallel losses of H3K9me3 and H3K27me3, but inhibition of viral DNA revealed a differential inhibition of H3K27 removal, demonstrating a functional separation of the two events. Therefore, by 6 hpi, the histones that remained bound to the *ICP8* region had a lower proportion of heterochromatin-modified H3 relative to *GAPDH* gene sequences. This argues that, after 4 hpi, heterochromatin is preferentially removed, indicating either (i) the selective removal of histones bearing heterochromatin modifications or (ii) the recruitment of specific demethylases to remove methyl groups from the remaining histones. Additionally, the dependence of H3K27me3 removal on viral DNA synthesis suggests that histone exchange during DNA replication could be an important mechanism for heterochromatin removal. Interestingly, the timing of heterochromatin reduction coincided with the derepression of E viral gene transcription. This argues that histone and heterochromatin reduction is an important mechanism by which HSV promotes E gene expression and the progression of lytic infection and could mediate the transition from IE to E viral gene expression (46, 55). While transcription could lead to chromatin removal, previous studies (17) have shown that chromatin removal on the *ICP8* gene can occur in the absence of transcription.

**Removal of H3K9 trimethylation.** The removal of the H3K9me3 heterochromatin marker from 3 to 7 h is dependent on ICP0 and independent of viral DNA synthesis. Because this was also apparent in transfected cells, this appears to be an intrinsic

function of ICP0. There is no evidence that ICP0 is a demethylase, so it must work through cellular functions. It will be important to determine if ICP0 requires other demethylases such as LSD1 to promote these chromatin changes. IFI16, a cellular antiviral protein which promotes the H3K9me3 modification on histones on the HSV IE *ICP4* gene promoter, is degraded by ICP0 (49). Possible mechanisms for the effect of ICP0 on histone acetylation are known, but it is not clear how ICP0 affects histone methylation. Furthermore, it is not known how ICP0 exerts its effects on the viral genome. It is conceivable that ICP0 targets specific host proteins that are bound to or localized near viral DNA, such as PML (41) or IFI16 (50). In any event, it appears that H3K9me3 exerts a silencing function on the early *ICP8* promoter and that ICP0 counters that effect. This likely represents the checkpoint that characterized the immediate early to early gene expression transition (56).

Reduction of H3K9 trimethylation on IE gene promoters in HFFs is known to require LSD1 (KDM1A) and KDM4A–D (23). KDM3A is required for *ICP8* expression in U2OS cells (42). Further studies are needed to define the host factors needed for H3K9me3 removal in HFF cells and the mechanism(s) by which ICP0 promotes these factors.

**Removal of H3K27 trimethylation.** The removal of the H3K27me3 modification is initially accomplished through nucleosome removal and is dependent on viral DNA synthesis from 4 to 6 hpi. Because ICP0 is required for expression of the E proteins involved in viral DNA synthesis, at least part of the requirement for ICP0 in promoting H3K27me3 removal is indirect. The requirement for viral DNA synthesis could be due to viral

DNA synthesis diluting out the histone association, or a late viral gene product could promote the demethylation. Removal of H3K27me3 could be part of the mechanism by which viral DNA replication promotes late gene expression. Thus, the removal of H3K27me3 could be related to activation of viral late genes upon viral DNA replication. ICP8 is expressed in the absence of viral DNA synthesis; thus, the H3K27me3 modification cannot exert a complete silencing effect because it remains on the ICP8 promoter until the genome is replicated. Interestingly, the KDM6A H3K27me2/3 demethylase is required for optimal ICP8-GFP expression in U2OS cells (42). Further studies are needed to determine the host factors needed for H3K27me3 removal in HFF cells. In eukaryotic cells, H3K9me3 is generally associated with constitutive heterochromatin and permanent repression, while H3K27me3 is associated with facultative heterochromatin that is characterized by repression with periodic activation, as seen in developmental genes and in bivalent domains in stem cells (57, 58). Further studies to define the complete range of histone modifications on viral chromatin on the ICP8 gene promoter are needed to understand the full function of the H3K27me3 histone modification.

These results argue that the H3K9 trimethylation and H3K27 trimethylation modifications are independent events. We have recently observed that during latent infection that there is an apparent inverse relationship between the H3K27me3 and H3K9me3 modifications (59). These results in combination provide further evidence for the complex nature of posttranslational modifications of histones and their role in epigenetics. The elucidation of the mechanisms by which the three stages of HSV chromatin modification defined here, namely, general removal of chromatin, removal of H3K9me3, and removal of H3K27me3, should identify additional targets for intervention against HSV infection.

## MATERIALS AND METHODS

**Plasmids, cells, and viruses.** Primary HFF, HeLa, Vero, U2OS, and 293T cells were obtained from the American Type Culture Collection (Manassas, VA). The *n212 ICP0* nonsense mutant virus contains a nonsense mutation in codon 212 (53). For this study, we constructed a corresponding ICP0<sup>+</sup> rescued virus, *n212R*, by homologous recombination of a full-length *ICP0* gene in linearized pCIΔLAT.full plasmid with infectious *n212* viral DNA. The *dProm* mutant virus and the *PromR* rescued virus were constructed by homologous recombination with *ICP0*-null 7134 infectious viral DNA (53) together with the pCIΔLAT.fullProm *ICP0* promoter mutant plasmid and the pCIΔLAT.full WT full-length *ICP0* plasmid, respectively. See the supplemental material for more details. We verified that the rescued viruses followed infection kinetics similar to those seen with WT HSV-1 KOS, which was also titrated on U2OS cells.

**Infections.** HFF cells were infected with viruses at 3 PFU/cell. For ChIP experiments testing the effects of inhibiting viral DNA synthesis, sodium phosphonoacetate (PAA) was added to the medium at 200 μg/ml along with 10 mM HEPES at the time of infection and maintained in the medium after infection until the cells were harvested at the indicated time points (17, 60, 61). See Text S1 (supplemental Materials and Methods) for more details.

**Cotransfection of *ICP0* and *ICP8-GFP* genes.** HEK 293T cells were seeded in 6-well plates to ensure <50% confluence on the day of transfection. Cells were cotransfected with 0.4 μg of pICP8-GFP (42) and either 0.4 μg of pICP0 (49) or 0.4 μg of an empty vector plasmid (pEV) using Effectene (Qiagen) according to the manufacturer's instructions. At 48 h after transfection, cells were harvested and processed for ChIP assays or for quantification of RNA expression.

**Chromatin immunoprecipitation.** ChIP experiments were carried out as previously described (17, 24, 62, 63), with some modifications. See Text S1 (supplemental Materials and Methods) for detailed descriptions of chromatin immunoprecipitation and quantification of viral gene expression.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02007-15/-/DCSupplemental>.

Text S1, DOCX file, 0.16 MB.

Figure S1, EPS file, 1.3 MB.

Figure S2, EPS file, 0.7 MB.

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